Online Supplement

Clonal Architecture of *CXCR4* WHIM-like Mutations in Waldenstrom's Macroglobulinaemia.

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Development of quantitative AS-PCR assays for $CXCR^{S338X}$ mutations

Since $CXCR4^{S338X}$ mutations can occur due to C>G and C>A mutations at nucleotide position 1013 in the CXCR4 gene, we developed two AS-PCR assays to permit their detection. Three reverse primers were designed to differentiate the nucleotide positions corresponding to the mutant and wild-type alleles of $CXCR4^{S338X}$. To optimize the specificity, an internal mismatch in the third position from the 3'-end was introduced. 5'-AGACTCAGACTCAGTGGAAACAGTTC-3' was used to detect the C>G mutation, and 5'-AGACTCAGACTCAGTGGAAACAGGTT-3' was used to detect the C>A mutation. The wild-type specific reverse primer was 5'-AGACTCAGACTCAGTGGAAACAGTTG-3'. The common forward primer was 5'-TTCCACTGTTGTCTGAACCCCATC-3'. Quantitative detection of the $CXCR4^{S338X}$ mutations was achieved using the above described primers with Power SYBR® Green PCR Master Mix used in accordance with manufacturers instructions for the ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, CA). PCR reaction was performed in a final volume of 25 µl with 25 nM of each primer and 50 ng DNA. Thermal cycling conditions were as follows: 10 min at 95^0C, followed by 40 cycles of 95^0 C for 15 seconds and 60^0

C for 60 seconds. Each sample was assayed in triplicate. The standard curves for the CXCR4^{S338X} mutations were generated by serial dilution of mutant DNA with wild-type DNA (50%, 10%, 2%, 0.4%, 0.08%, and wild-type). For the corresponding reference PCR, the forward and reverse primers were 5'-ACTACATTGGGATCAGCATCGACTC-3' and 5'-TGAAGACTCAGACTCAGTGGAAACAG-3', respectively. The mutation burden was calculated based on the value of delta C_T generated from the standard curves.

The real-time AS-PCR developed for the CXCR4$^{S338X\ C>A}$ variant detected this mutation at a dilution of 0.4% with a $\geq$ 2 cycle difference (cutoff of 9.1) from the wild-type DNA background. The correlation coefficient of the standard curve for this assay was 0.992 and demonstrated a slope value of -3.45 (**Figure 1**). The melting curve analysis revealed that the CXCR4$^{S338X\ C>A}$ mutant-specific amplicon melted at 81.2^{0}C. For the CXCR4$^{S338X\ C>G}$ variant, real-time AS-PCR detected this mutation at a dilution of 0.16% with $\geq$ 2 cycle difference (cutoff of 10.5) from the wild-type DNA background. The correlation coefficient of the standard curve for this assay was 0.999 with a slope value of -3.74 (**Figure 1**). The melting curve analysis revealed that the CXCR4$^{S338X\ C>G}$ mutant-specific amplicon melted at 81.7^{0}C.

We first analyzed CD19-sorted BM and PB samples using the CXCR4$^{S338X\ C>A}$ AS-PCR assay in 13 (4 CXCR4$^{S338X\ C>A}$; 4 CXCR4$^{S338X\ C>G}$; 5 CXCR4^{WT}) WM patients whose CXCR4 status was determined by Sanger sequencing of BM

CD19-sorted cells, and PB CD19-sorted cells from 13 healthy donors. Sanger sequencing was used to confirm CXCR4 wild-type status in healthy donors. Healthy donors displayed a median ΔC_T value of 11.4 (range 11.1-12.4 cycles), whereas CXCR4$^{S338X\ C>A}$ WM patients had a median ΔC_T value of 0.8 (range 0.3 to 1.5 cycles) in BM samples and a median ΔC_T value of 4.0 (range 1.2-7.3 cycles) in PB samples; $p<0.001$ for BM and PB sample comparisons to healthy donor samples. Among the CXCR4^{WT} and CXCR4$^{S338X\ C>G}$ patients, the median ΔC_T values with this assay were 10.8 (range 9.9 to 12.3 cycles) and 12.2 (range 11.2 to 13.5 cycles) in BM samples, respectively ($p<0.01$ for comparisons of both cohorts versus CXCR4$^{S338X\ C>A}$ WM patient BM samples). For PB samples, the median ΔC_T values for CXCR4^{WT} and CXCR4$^{S338X\ C>G}$ patients were 10.6 (range 10.0 to 11.2 cycles) and 12.1 (range 11.2 to 13.5 cycles); $p\leq0.01$ for comparisons of both cohorts versus CXCR4$^{S338X\ C>A}$ WM patient BM and PB samples. There were no significant differences in median ΔC_T values for either CXCR4^{WT} or CXCR4$^{S338X\ C>G}$ patient BM or PB samples versus healthy donors. While CXCR4$^{S338X\ C>A}$ was detected in 4/4 (100%) BM samples of patients bearing this variant by Sanger sequencing, it was detected in only 1/4 (25%) PB samples collected simultaneously in these patients. In contrast, using a ΔC_T cutoff of 9.1 that represented a 2 cycle difference from the lowest healthy donor cutoff, CXCR4$^{S338X\ C>A}$ was positive in BM and PB samples from all 4 of these patients.

We next analyzed BM and PB CD19-sorted samples using the CXCR4$^{S338X\ C>G}$ AS-PCR assay that we developed in the same 13 WM patients and 13 healthy

donors described above. Healthy donors displayed a median ΔC_T value of >17.0 cycles, whereas $CXCR4^{S338X\ C>G}$ WM patients had a median ΔC_T value of 0.8 (range 0.2 to 1.4 cycles) in BM samples and a median ΔC_T value of 1.6 (range 0.9-2.6 cycles) in PB samples; $p<0.001$ for BM and PB sample comparisons to healthy donor samples. Among $CXCR4^{WT}$ and $CXCR4^{S338X\ C>A}$ patients, the median ΔC_T values with this assay were >11.9 cycles and 11.2 (range 11.1 to 11.4 cycles) in BM samples, respectively ($p<0.01$ for comparisons of both cohorts versus $CXCR4^{S338X\ C>G}$ WM patient BM samples). For PB samples, the median ΔC_T values for $CXCR4^{WT}$ and $CXCR4^{S338X\ C>A}$ patients were >14.2 cycles; and 11.2 (range 11.1 to 11.4 cycles); $p\leq0.01$ for comparisons of both cohorts versus $CXCR4^{S338X\ C>G}$ WM patient BM and PB samples. There were no significant differences in median ΔC_T values for either $CXCR4^{WT}$ or $CXCR4^{S338X\ C>A}$ patient BM or PB samples versus healthy donors. $CXCR4^{S338X\ C>G}$ was detected in 4/4 (100%) BM and simultaneously collected PB samples of patients bearing this variant by Sanger sequencing. Using a ΔC_T cutoff of 10.5 representing a >2 cycle difference from the lowest healthy donor cutoff, $CXCR4^{S338X\ C>G}$ was positive in BM and PB samples from all 4 of these patients.

Supplementary Figure 1. Sensitivity and specificity plots for real-time AS-PCR assays. (A) Delta reaction curve for real time AS-PCR assays for $CXCR4^{S338X\ C>A}$ and $CXCR4^{S338X\ C>G}$. Serial dilutions of DNA from malignant cells isolated from patients with $CXCR4^{WT}$ against those from patients with either $CXCR4^{S338X\ C>A}$ or $CXCR4^{S338X\ C>G}$ were made at the concentrations indicated in the amplification plots. The $CXCR4^{S338X\ C>A}$ allele was detected to a dilution of 0.4%, and the $CXCR4^{S338X\ C>G}$ allele was detected to a dilution of 0.16%. (B)

Standard curves for CXCR4$^{S338X\ C>A}$ and CXCR4$^{S338X\ C>G}$ AS-PCR assays. The correlation coefficients and slope values for the assays are shown. (C) Dissociation curves for real time AS-PCR assays for CXCR4$^{S338X\ C>A}$ and CXCR4$^{S338X\ C>G}$.

Supplementary Figure 1.